

## Hepatic and renal D-amino acid oxidase activities in the growing rat after ten days of protein undernutrition and refeeding

S Carreira, D Brun-Achirou, P Brachet, A Puigserver \*

*Laboratoire de biochimie et biologie de la nutrition CNRS-Ura 1820,  
Faculté des sciences de Saint-Jérôme, Service 342, avenue Escadrille-Normandie-Niemen,  
13397 Marseille cedex 20, France*

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**Summary** — The activity levels of hepatic and renal D-amino acid oxidase (EC 1.4.3.3), a key enzyme for D-amino acid utilization in mammals, were determined in growing rats after a 10 day period of protein undernutrition and subsequent refeeding. Reducing the protein intake for 10 days (3% casein diet) resulted in an 8% loss of the animals' mean body weight and a 50% decrease in the mean size of the liver and kidney as compared to the control animals fed on a 22% casein diet during the same period. When the undernourished rats were refed with the normal protein diet, their weight increased about four fold as compared with that of the control animals, and after a ten day period of refeeding, the lost body and tissue weights were completely recovered. As far as the specific activity of D-amino acid oxidase was concerned, a 44% reduction took place in the liver of rats subjected to protein undernutrition for 10 days. During the period of refeeding, however, the enzyme activity level increased slowly in comparison with the overall hepatic protein level, since its specific activity on day 10 was still 28% below that of the control rats. In sharp contrast, no significant change in the kidney enzyme level was observed throughout these nutritional manipulations. This study strongly suggests that the synthesis and/or catabolism of D-amino acid oxidase may depend on the dietary protein content of the liver but not on that of the kidney. This suggests that D-amino acid oxidase may possibly play distinct physiological roles in these two body organs.

**rat / undernutrition / refeeding / D-amino acid oxidase / liver / kidney**

**Résumé** — Conséquences d'une malnutrition protéique suivie d'une réalimentation équilibrée sur les niveaux d'activité de la D-aminoacide oxydase hépatique et rénale chez le jeune rat. Le but de ce travail a été de déterminer, chez le rat en croissance, les effets d'une malnutrition protéique de 10 jours suivie de différentes périodes de réalimentation sur les niveaux d'activité hépatique et rénale de la D-aminoacide oxydase (EC 1.4.3.3) dont le rôle est déterminant pour l'utilisation métabolique des D-aminoacides d'origine alimentaire. L'administration d'un régime contenant 3% de caséine pen-

\* Correspondence and reprints

*durant 10 jours entraîne une diminution d'environ 8% de la masse corporelle et d'environ 50 % de celles du foie et des reins, comparativement aux rats témoins recevant un régime à 22% de caséine. La prise pondérale chez les rats renutris est sensiblement quatre fois plus rapide que celle de rats témoins du même âge. Dix jours de réalimentation suffisent pour que les masses considérées aient des valeurs proches de celles des témoins. L'activité spécifique de la D-aminoacide oxydase hépatique diminue de 44 % au bout de 10 jours de malnutrition et ne s'accroît que lentement du fait de la réalimentation, alors que le contenu protéique total du tissu redevient très rapidement quasi normal. Au 10<sup>e</sup> jour de réalimentation le niveau de l'enzyme est encore inférieur de 28% à celui des témoins. L'enzyme rénale, en revanche, n'est affectée par aucune des situations nutritionnelles imposées. Nos résultats suggèrent que la biosynthèse et/ou le catabolisme de la D-aminoacide oxydase hépatique, contrairement à l'enzyme rénale, sont modulés par la teneur en protéines du régime, ce qui soulève la possibilité de fonctions physiologiques distinctes pour chacune de ces deux enzymes.*

**rat / malnutrition / réalimentation / D-aminoacide oxydase / foie / rein**

## INTRODUCTION

The biological utilization of dietary D-amino acids in mammals depends mainly on the presence of D-amino acid oxidase (D-AAOX, EC 1.4.3.3) in several tissues (Baker, 1986). D-AAOX is a flavoenzyme which catalyzes the oxidative deamination of most of the neutral D-amino acids to the corresponding  $\alpha$ -keto acids. Although enzymatic activity of this kind has long been known to occur in mammalian tissues (Krebs, 1935), the enzyme has only quite recently been purified and crystallized, mainly from pig kidney, and its enzymic and physico-chemical properties well documented (Bright and Porter, 1975; Ronchi et al, 1982; Fukui et al, 1988; Pollegioni et al, 1994). The question concerning the physiological role of D-AAOX remains to be determined, however, since as far as we know, no biosynthetic pathway for neutral D-amino acids has yet been found to exist in a variety of mammalian tissues. In addition, the alternative proposal that D-AAOX might have substrates other than D-amino acids (Hamilton, 1985) has so far not been substantiated at either the physiological or the biochemical levels.

Protein deprivation is known to markedly affect the specific activity of a number of enzymes involved in the catabolism of amino acids in both the liver and kidney of the growing rat (Freedlang and Szepesi, 1971).

Although several studies have been carried out on the effects of dietary protein on D-AAOX, the results are rather contradictory and have not shed any light on the biological role of the enzyme. This may be because of differences in the physiological states of the animals used and/or between the diets on which they were fed, in addition to the fact that the results were expressed differently. Some data suggested for instance that the enzyme activity level in the rat liver, but not in the kidney, was in direct proportion to the dietary protein level (Lardy and Feldott, 1950; Hoch-Ligeti, 1953), whereas in the studies by Vanderlinde and Westerfeld (1950), on the contrary, no variations were ever observed in either type of tissue. On the other hand, the total activity of renal D-AAOX was found by Iacobellis et al (1954) to decrease significantly in animals which were fed on a low protein diet, but to remain practically unchanged when expressed with respect to the tissue nitrogen.

Since all the possible changes in tissue D-AAOX activity are of prime importance to the eventual understanding of the nutritional utilization of dietary D-amino acids by growing animals, we decided to investigate the effects of protein undernutrition on the enzyme's activity in both the liver and the kidney of growing rats, using a new enzyme assay procedure. Contrary to what was done in all the studies mentioned above,

short-term experiments lasting no longer than ten days were carried out and the time related effects of refeeding with a normal diet were also subsequently investigated.

to the period of refeeding. The compositions of the diets used are shown in table I. The food consumption was measured daily, while the rats' body weights were monitored three times a week.

## MATERIALS AND METHODS

### *Animals and diets*

Weaned male, Wistar rats (Iffa-Credo, L'Arbresle, France) were used throughout this study. They were housed individually in a temperature-controlled room on a 12 h dark/light illumination cycle and had free access to food and water. All the animals were fed on a commercial standard pelleted diet from Usine d'Alimentation Rationnelle, UAR (Villemoisson-sur-Orge, France) for 5 days to compensate for any differences between the animals' previous dietary histories. The rats were then randomly distributed into 11 groups of at least five animals each, with body weights ranging from 75 to 90 g. They were given a semisynthetic powdered diet. Two groups of rats were fed on a 22% casein control diet for 10 or 20 days, while the other nine groups were first given the 3% casein experimental diet for 10 days. After this period of protein undernutrition, eight out of the nine groups were given the control diet for 1, 2, 4, 5, 6, 7, 8 or 10 days further, corresponding

### *Solubilization of D-AAOX from rat tissues*

At the end of each feeding period, the rats were sacrificed by cervical dislocation and their liver and kidney were rapidly excised. Once the liver had been completely bled by performing an in situ perfusion through the portal vein with an ice-cold 0.9% NaCl solution, both sorts of tissues were weighed and stored at  $-20^{\circ}\text{C}$  until use. Solubilization of peroxisomal D-AAOX was carried out at  $4^{\circ}\text{C}$  using a modified version of the procedure previously described by Gavazzi et al (1987). The thawed tissues were homogenized with a Polytron homogenizer for 30 s in 3 vol (v/w) of a 50 mM tetrasodium phosphate buffer (pH 8.5) containing 10  $\mu\text{M}$  flavin adenine dinucleotide (FAD), 1 mM dithiothreitol (DTT) (Boehringer, Mannheim, Germany), 1 mM benzamidine and 1 mM phenylmethanesulfonyl fluoride (PheMeSO<sub>2</sub>F) (Fluka, Buchs, Switzerland). This step was repeated five times at 30 s intervals in ice, and the resulting homogenate was centrifuged at 100 000 g for 40 min. Enzyme assays were performed either directly on the supernatant (kidney) or after dialyzing the solution twice, first for 2 h and then overnight against 100 vol of the

**Table I.** Composition of the diets (g/100 g).

Component <sup>a</sup>	Diet		
	Standard	Control	Experimental
Casein	23	22	3
Starch	58	63	83.9
Sunflower oil	3	3	0.4
Mineral mix	6	6	6
Vitamin mix	1	1	1
Cellulose	6	5	5.7
Sodium bicarbonate	3	—	—

<sup>a</sup> All the components were from UAR (Villemoisson-sur-Orge, France), except the oil, which was from Nouvelles Huileries et Raffineries Massilia, NHRM (Marseille, France). The control diet was also used for refeeding the rats after they had been fed the experimental diet corresponding to the protein undernutrition conditions.

pyrophosphate buffer containing 1 mM benzamide only (liver). FAD, PheMeSO<sub>2</sub>F and DTT were then added to the dialysate to final concentrations of 10 µM in FAD and 1 mM in the others, in order to prevent any denaturation of the enzyme. The rationale for treating liver and kidney samples differently before performance of the enzymatic assays is to abolish a lag usually observed with the corresponding supernatant from liver homogenate.

### **Assay to determine D-AAOX specific activity**

D-AAOX activity was determined spectrophotometrically by a modified version of the method described by Nagata et al (1988). The product 2-keto 4-methylthiobutanoate (KMB), which results in the enzymatic oxidation of the substrate D-methionine, was detected at 445 nm as the hydrazone derivative. Thirty microlitres of crude liver dialysate or tenfold diluted kidney supernatant was mixed in a 1 mL spectrophotometer cuvette with 50 µL H<sub>2</sub>O, 40 µL of 100 mM D-methionine and 80 µL of a 127 mM pyrophosphate buffer (pH 8.5) containing 10 µM FAD and 600 µg/mL catalase (Boehringer, Mannheim, Germany). The mixture was then allowed to stand at 37 °C for various incubation periods. Catalase was included in the medium so as to eliminate the H<sub>2</sub>O<sub>2</sub> formed in the oxidase reaction, which might have had inhibitory effects on the reaction. The reactions were stopped by adding 100 µL of 1 mM 2,4-dinitrophenylhydrazine dissolved in 1 N HCl to the contents of the cuvette. Crystallization of the hydrazone derivative was performed after a further 10 min incubation at 37 °C. The hydrazone crystals were dissolved by adding 700 µL of 0.6 N NaOH and allowing the mixture to stand for 5 min at room temperature. The absorbance of the sample was measured at 445 nm against a blank, processed as usual but containing no D-methionine. The D-AAOX specific activity (U/mg) was expressed as nmol of KMB formed per min and per mg of protein according to the formula:

$$[U/mg] = [D(OD)] \times [e] \times [1/v] \times [1/PC] \times [1/t]$$

where D(OD) stands for the optical density at 445 nm of the incubated sample, corrected for its absorbance at zero time. This was obtained by adding the enzyme fraction and 2,4-dinitrophenylhydrazine simultaneously.

e stands for the molar absorption coefficient of the 2,4-dinitrophenylhydrazone of KMB. It was derived from the slope of a standard curve for the α-keto acid (nmol/OD units) obtained in the following way. Various amounts (5–25 nmol) of KMB were dissolved in 200 µL of a 50 mM pyrophosphate buffer (pH 8.5), mixed with 100 µL of 1 mM 2,4-dinitrophenylhydrazine in 1 N HCl and processed as with a normal tissue sample.

v and PC are the volume (mL) and the protein concentration (mg/mL) of the sample. The latter was determined using the method of Hartree (1972) with bovine serum albumin as standard.

t is the incubation period (min). It was included in the portion of the time course where the initial reaction rates occurred.

### **Statistical analysis**

The results were expressed as means with their standard deviations. Statistical significance was assessed using the Student's *t*-test for unpaired data. The differences were taken to be significant at *P* < 0.05.

## **RESULTS**

### **Food intake and weight determinations**

Significant differences were observed in feed intake between the animals fed on the experimental diet and those in the two control groups. The individual daily food intake ranged from 10 to 17 g in the former group and from 15 to 20 g in the other two groups, and amounted to 20–26 g when the control diet was resumed.

The changes which were observed in the body weights of the rats fed on the experimental diet (protein undernutrition conditions) for a 10 day period and the subsequent refeeding periods are shown in table II. Consumption of the low-protein, low-fat diet put an immediate stop to the animal's growth. This was followed by substantial weight loss and eventually by a relative con-

**Table II.** Effects of 10 day protein undernutrition and subsequent refeeding on the mean body weight (g) of rats.

Group	Undernutrition		Refeeding	
	Initial weight	Final weight	Day	Final weight
1	90.3 ± 4.6	82.2 ± 3.4	0	—
2	88.4 ± 4.1 <sup>a</sup>	80.5 ± 2.8	1	93.9 ± 3.4 <sup>a</sup>
3	86.7 ± 3.5	78.0 ± 2.6	2	98.2 ± 3.7
4	87.1 ± 2.8	77.0 ± 5.4	4	106.6 ± 15.5
5	85.3 ± 4.0	76.1 ± 2.9	5	121.9 ± 7.1
6	89.8 ± 6.6	82.9 ± 1.0	6	139.8 ± 2.7
7	84.5 ± 1.5	81.7 ± 1.6	7	138.1 ± 3.9
8	80.6 ± 4.2 <sup>a</sup>	78.8 ± 3.9 <sup>a</sup>	8	137.9 ± 10.2
9	85.2 ± 0.4	81.0 ± 2.5	10	161.7 ± 8.1

Values are mean body weights ± SD of five rats in each experimental group. Data with the same superscripts on the same horizontal line were not significantly different ( $P > 0.05$ ).

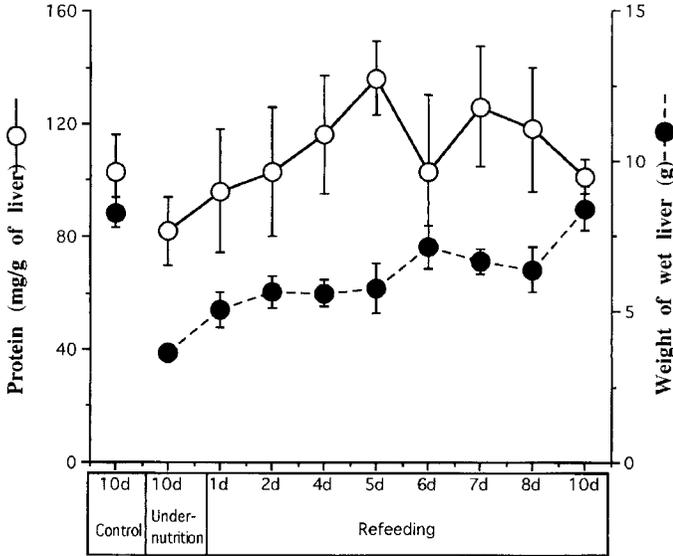
stant weight on the third or fourth day. The final mean weight of the rats ( $N = 40$ ) that were given the low-protein diet for 10 days was only  $92 \pm 3\%$  of their initial weight and  $53 \pm 2\%$  of that of the animals fed on the control diet during the same period ( $N = 5$ ). A rapid weight gain (eg,  $13 \pm 1$  g/rat after the first day) was observed after the readministration of the control diet to the undernourished rats. On the last day of the refeeding period (tenth day), their average body weight was only 6.8% lower than that of the control rats ( $173.4 \pm 12.5$  g), which had been fed on the 22% casein diet for 20 days. The difference between these two groups was not found to be significant at that time. It is worth mentioning that during the refeeding period, the daily weight gain of the experimental animals ( $9.1 \pm 1.9$  g) was almost four times that of the controls ( $2.5 \pm 0.3$  g).

Compared to the control nutrition conditions, feeding on the low-protein, low-fat diet for 10 days resulted in a 50% decrease in the weights of both the liver (fig 1) and the kidney (fig 2) of the animals. In the protein-deprived animals, which were refed with the

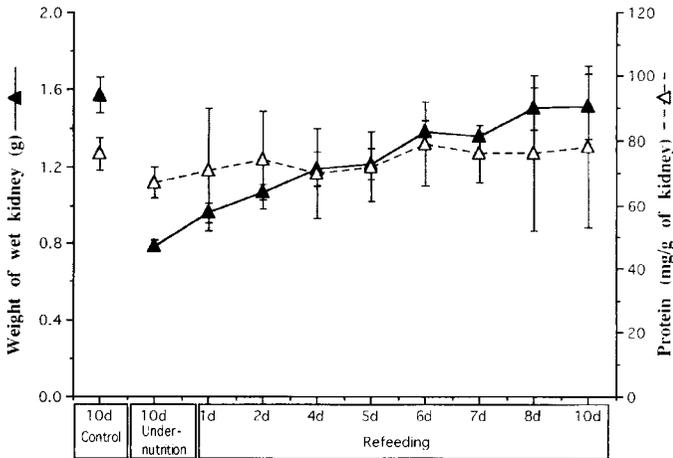
control diet after the 10 day period of undernutrition, the normal size of both organs was practically completely recovered by the end of the refeeding period. The overall protein level in the liver of the undernourished rats ( $82 \pm 12$  mg/g of tissue,  $N = 5$ ) was 80% ( $P < 0.05$ ) that of the control rats ( $103 \pm 13$  mg/g of tissue,  $N = 5$ ). A normal protein level was reached as early as the first day on which the undernourished rats were refed with the control diet, and a significant level of protein accumulation was observable even on the fifth day. On the other hand, there was no significant difference between the kidney protein contents of the rats in the control and experimental groups despite the 50% decrease in the weight of the kidney observed at the end of the period of undernutrition.

#### ***D-AAOX activity in rat liver and kidney***

The effects on the liver and kidney D-AAOX activities of maintaining the rats on a low-protein diet for 10 days followed by increas-



**Fig 1.** Effects of a 10 day period of protein undernutrition and subsequent refeeding on the weight (●) and protein content (○) of rat liver. Points and bars are the means and SD, respectively, of at least five determinations carried out with different animals. When not indicated, the SD were smaller than those indicated by the symbols.

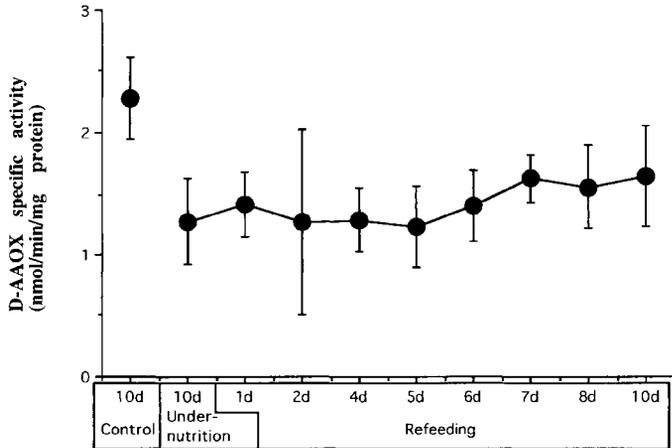


**Fig 2.** Effects of a 10 day period of protein undernutrition and subsequent refeeding on the weight (▲) and protein content (△) of rat kidney. See figure 1 for further information.

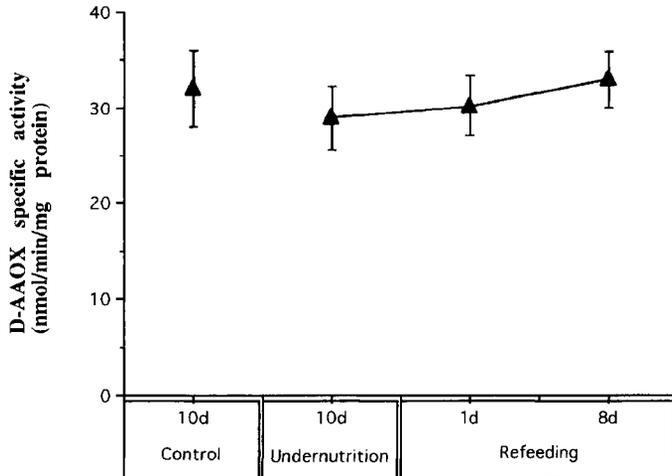
ing periods of refeeding with the control diet are shown in figures 3 and 4, respectively. Strong variations in the enzyme-specific activity levels were observed, depending on the tissue. The liver D-AAOX specific activity decreased by 44% in the undernourished rats in comparison with the normally fed animals, whereas only a 10% (non-significant)

activity loss was observed in the case of the kidney enzyme. During the period of refeeding, no change was observed in the kidney D-AAOX activity level, and only a slight increase in that of the liver enzyme, since its specific activity remained 28% below that recorded in the control rats on the tenth day of refeeding.

**Fig 3.** Effects of a 10 day period of protein undernutrition and subsequent refeeding on liver D-amino acid oxidase (D-AAOX) activity. See figure 1 for further information.



**Fig 4.** Effects of 10 day protein undernutrition followed by normal refeeding on kidney D-amino acid oxidase (D-AAOX) activity. See figure 1 for further information.



**DISCUSSION**

These experiments demonstrated a marked decrease in the specific activity of D-AAOX in the liver of growing rats maintained in a protein and fat undernutritive condition for a 10 day period. This result was not observed in the kidney. These data are in agreement with previous studies in which rats were given low-protein diets for longer periods of time (Lang, 1947; Lardy and Feldott, 1950;

Hoch-Ligeti, 1953). Although the data from these studies and our own results are not directly comparable, it can nevertheless be noted that a comparable degree of enzyme inhibition was reached, averaging 50%. This strongly suggests that the D-AAOX activity no longer decreased after day 10 of undernutrition. Furthermore, our data clearly indicate that the liver activity level of D-AAOX, known to be twice as high in the periportal hepatocytes than in the perivenous hepa-

toocytes (Le Hir and Dubach, 1980), decreased more rapidly than the overall tissue protein level in the undernourished rats as compared to the control rats. Upon refeeding with the 22% casein diet for 10 days, the enzyme specific activity still remained significantly below that measured in the control animals. The observed decrease in the enzyme activity may have resulted from decreased synthesis and/or increased catabolic rates, although direct data on both points are still lacking, and the mechanisms involved under each nutritional condition, namely undernutrition and refeeding, might be different. In particular, refeeding is known to induce cell proliferation in the liver, generally concomitantly with a decrease in D-AAOX level in comparison with that of normally fed control rats (Mochizuki et al, 1976; Sun and Cederbaum, 1980). These observations could explain the slow resumptions of the enzyme activity observed in the refed animals.

Comparisons between the composition of the low-protein diet and that of the control diet suggest that the observed changes in D-AAOX activity might not have resulted simply from the reduced protein substrate availability, since increasing the starch content or decreasing the oil content in the experimental diet was not found to change the enzyme activity in either the rat liver or kidney (data not shown). Furthermore, dietary casein contains very small quantities of D-amino acids (Man and Bada, 1987) and consequently, the administration of a 3% casein diet instead of a 22% casein diet cannot, therefore, have substantially reduced the intake of D-amino acids by the undernourished rats. In humans, senescence and some pathological situations such as renal disease have been found to increase the plasma levels of D-amino acids (Nagata et al, 1987). In addition, in mutant mice lactating D-AAOX, D-amino acids seem to be concentrated in many tissues, including the liver and kidney (Nagata et al, 1989; Nagata et al, 1994). Whether or not D-amino acids

are the effectors of the enzyme activity in animal tissues still remains to be established. Meister et al (1960) and more recently Wellner (1971) reported that feeding D-amino acids to mice did not affect the D-AAOX activity, while Lyle and Jutila (1968) obtained exactly the opposite result. Any changes in the intracellular concentration of D-amino acids, which may occur as the result of protein undernutrition, actually seem unlikely, in our opinion, to result in significant variations in D-AAOX activity.

Of course the rapid decrease in both the liver and kidney weights as well as in the body weight of young rats subjected to protein undernutrition was not unexpected, but the supranormal rates of weight gain observed in response to refeeding the rats with a 22% casein diet provided interesting information about the ability of these animals to recover a normal physiological state. Although the increased food intakes may account for the enhanced rates of weight gain, evidence was also obtained in the present study that complex metabolic changes may have occurred in the refed animals. It clearly emerged that the weight gain per unit of food consumed was significantly higher in the refed animals than in the controls, which is in line with previous data (Levitsky et al, 1976; Boyle et al, 1978), proving that rats were able to utilize food more efficiently after a period of protein restriction than when they were fed normally. The mechanisms responsible for this apparently enhanced energy utilization in the young rat remain to be elucidated, however, although both a drop in the metabolic rate and a decrease in the energy dissipated in the form of heat might be involved.

Our results also indicate that during the period of protein undernutrition, both the liver and kidney rapidly supplied amino acids to the rest of the animals' body by mobilizing their endogenous labile protein stores. Unlike that in the kidney, the protein level in the liver as expressed per unit of wet

organ weight decreased by 20%, which suggests that the weight loss of the tissue per se was not entirely responsible for the decrease in its protein content. Dietary protein deprivation, therefore, seems to lead to a more rapid breakdown of the reserve protein stores in this tissue than in the case of any of the other dietary components. With the resumption of the growth of the undernourished rats upon refeeding with the control diet, the hepatic protein level rapidly returned to a normal value. A transient peak phase even seemed to occur between days 4 and 8, indicating that a considerable accumulation of protidic material had occurred in the liver of the refed animals.

The present results show that, unlike liver, the kidney of undernourished and subsequently refed rats exhibited a remarkably stable level of D-AAOX as compared with that of the whole tissue proteins. As the renal D-AAOX is not affected by the modulations in nutritional conditions, this appears to be consistent with the role attributed to this enzyme and with its localization in the kidney proximal tubules (Ohno, 1985). Although direct evidence is still lacking, D-AAOX is possibly involved in the renal reabsorption processes, as suggested by the fact that a lack of D-AAOX activity in mutant mice resulted in a marked aminoaciduria (Konno et al, 1988; Nagata et al, 1989). During protein undernutrition, the preservation of both D-AAOX activity and nutrient reabsorption in kidney epithelial cells may markedly reduce the body energy expenditure of rats. In agreement with our previous studies (Brachet et al, 1991), in which rats fed a 25% sunflower seed oil containing diet exhibited a strong decrease in hepatic D-AAOX specific activity as compared to those fed equal amounts of hydrogenated coconut oil for the same 5 or 10 day period while kidney D-AAOX specific activity was unchanged under both experimental conditions, we conclude that nutritional manipulations affect the liver and kidney D-amino acid oxidases differently. These observa-

tions can probably be related to different physiological functions of the D-amino acid oxidases in the liver and kidney.

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